Targeting signals for a bacterial Sec-independent export system direct plant thylakoid import by the ΔpH pathway

Margaret Wexler[a,b,c], Erik G. Bogsch[d], Ralf Bernd Klösgen[e], Tracy Palmer[b,c], Colin Robinson[d], Ben C. Berks[a,b,*]

[a] *Centre for Metalloprotein Spectroscopy and Biology, University of East Anglia, Norwich NR4 7TJ, UK*
[b] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[c] *Nitrogen Fixation Laboratory, John Innes Centre, Norwich NR4 7UH, UK*
[d] *Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*
[e] *Botanisches Institut der Ludwig-Maximilians-Universität, Menzinger Strasse 67, 80638 Munich, Germany*

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Abstract Preproteins targeted to the Sec-independent protein transport systems of plant thylakoids and of bacteria both have unusual transfer peptides bearing a consensus twin-arginine motif. Possible mechanistic similarity between the two Sec-independent transport pathways was investigated by assessing the ability of bacterial twin-arginine transfer peptides to direct thylakoid import. High efficiency import was observed. This process was demonstrated to occur specifically via the Sec-independent ΔpH pathway and to depend on an intact twin-arginine motif on the transfer peptide. These results provide strong evidence for the operation of mechanistically related Sec-independent protein transport pathways in chloroplasts and bacteria.

Key words: Sec-independent; Bacterial protein export; Thylakoid import; Twin-arginine transfer peptide; Signal peptide

1. Introduction

Proteins that require translocation across biological membranes are normally synthesised with amino-terminal targeting signals. Thus in Gram-negative bacteria many periplasmic proteins are synthesised with cleavable 'signal' peptides that direct translocation by the well-defined Sec pathway. In this system, which involves both soluble cytoplasmic and integral membrane proteins, transport is driven primarily by ATP hydrolysis [1]. Bacterial signal peptides typically comprise three distinct domains: an amino-terminal basic domain, a hydrophobic core domain and a more polar C-terminal domain ending with small side-chain residues prior to the cleavage site for the signal peptidase [1–3]. The available evidence indicates that a system with broad similarity to the Sec apparatus also operates to translocate proteins across the thylakoid membrane of chloroplasts [4–6]. Substrates for this system bear Sec-like signal peptides.

Recent studies have pointed to the operation of mechanistically novel Sec-independent protein translocation systems in both bacteria and chloroplasts. In chloroplasts a distinct subset of thylakoid lumenal proteins are imported by a pathway that requires neither ATP nor soluble stromal factors, but which is instead completely reliant on the ΔpH gradient across the thylakoid membrane [7–11]. Remarkably, the targeting signals of these proteins strongly resemble Sec-pathway signal peptides in overall structure, yet are able to direct translocation by only the ΔpH-dependent pathway [12,13]. A critical factor in sorting to the ΔpH pathway is the presence of a twin-arginine motif immediately prior to the hydrophobic core domain in these peptides (*transfer peptides*; [14] and Fig. 1).

There are now indications that a related system may exist in bacteria. The first component of the chloroplast ΔpH-dependent translocase was recently identified [15] and homologues of this protein are coded by a wide range of bacteria. Further, a distinct subset of periplasmic proteins, the majority of which bind cofactors, are synthesised with targeting signals that strongly resemble thylakoid twin-arginine transfer peptides ([16] and Fig. 1). Notably, one protein with this type of targeting signal has recently been shown to be exported in a Sec-independent manner [17]. These observations suggest that bacteria possess a protein export system that is structurally and mechanistically related to the Sec-independent pathway of thylakoid membranes, that plausible substrates of this pathway are the preproteins with twin-arginine transfer peptides and, given the evidence that cofactor insertion occurs before export [16,17], that the pathway may allow export of folded proteins.

In this report the possible mechanistic similarity between the bacterial and chloroplast Sec-independent transport pathways is investigated by testing whether bacterial twin-arginine transfer peptides can direct thylakoid import by the ΔpH-dependent pathway.

2. Materials and methods

The plasmids encoding chimeric proteins were based on pBluescript KS (Stratagene Inc.). The inserts were prepared by PCR amplification of the region coding for the bacterial presequences and ligation in-frame in front of the mature coding region of mature spinach 23K [18]. The R5K mutation in the FdnG transfer peptide was produced by oligonucleotide-directed mutagenesis as described [19].

Precursors were prepared by transcription in vitro followed by translation in a wheat germ cell-free lysate in the presence of [^{35}S]methionine as previously described [7].

Assays for the import of proteins into isolated, washed pea thylakoids were essentially as described in [20]. Assay mixtures containing 45 μl thylakoids (0.5 mg/ml chlorophyll) and 5 μl translation mixture were incubated for 20 min at 25°C, after which samples were analysed directly or after washing of the thylakoids and treatment with 200 μg/ml thermolysin. Where appropriate, incubations were pretreated with apyrase (1 unit) as described in [21], nigericin (4 μM) plus KCl (10 mM), or carried out in the presence of saturating concentrations (4 μM) over-expressed pea pre-23K as described in [22].

*Corresponding author. Fax: (44) (1603) 592250.
E-mail: b.berks@uea.ac.uk

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3. Results and discussion

The twin-arginine transfer peptides of two *Escherichia coli* proteins, TorA and FdnG, were selected for use in our thylakoid import experiments. TorA is the molybdopterin cofactor-containing enzyme trimethylamine *N*-oxide reductase [23]. This protein was previously shown to be exported by a Sec-independent mechanism in *E. coli* [17]. FdnG is the catalytic subunit of formate dehydrogenase-*N*. FdnG binds a molybdopterin cofactor together with an iron-sulphur cluster [24]. The TorA and FdnG transfer peptides each possess five of the six amino acids of the bacterial S/T-R-R-x-F-L-K consensus motif [16] (Fig. 1). An important difference between the two is that, while the FdnG transfer peptide contains the consensus lysine, the TorA transfer peptide resembles those of chloroplasts in lacking a charged residue at this position.

The full-length precursors of TorA and FdnG are poor subjects for our thylakoid import assay because they are relatively large (85 and 116 kDa, respectively), which makes it difficult to judge when the presequence is removed. It is also possible that these proteins would only be translocated if cofactor has been bound. We therefore simplified our experimental system by fusing each complete presequence, together with the first two amino acids of the mature protein, to mature 23 kDa protein (23K) of the plant photosystem II oxygen-evolving complex. 23K was chosen as a passenger protein because it does not bind a cofactor and is normally transported by, and is thus compatible with, the ΔpH-dependent pathway [7,8]. Because the signal peptidase recognition sites of bacteria and chloroplasts are similar [3,25] we expected that the thylakoid signal peptidase would be able to process the chimeric TorA-23K and FdnG-23K proteins.

Thylakoid import assays using the chimeric test preproteins are shown in the middle and lower panels of Fig. 2. Parallel control experiments with authentic plant pre-23K are shown in the upper panel. Both TorA-23K and FdnG-23K were processed by thylakoids to a smaller form (Fig. 2, compare lanes Tr and T). These mature forms, unlike the precursor proteins, are resistant to added protease (Fig. 2, compare lanes − and + therm.) demonstrating that the mature proteins have been transported into the thylakoid lumen. Under our assay conditions import of the chimeric preproteins is highly efficient, in some cases reaching 100% (e.g. Fig. 3A), and was never significantly different from that of authentic pre-23K. 23K lacking a transfer peptide is not imported into thylakoids (data not shown). Taken together these results demonstrate that bacterial transfer peptides are capable of directing efficient translocation of proteins across the plant thylakoid membrane.

We next investigated whether import of the chimeric constructs was mediated by the ΔpH-dependent pathway, by the Sec pathway or by a combination of the two mechanisms. Import of proteins by the Sec mechanism is totally dependent on ATP hydrolysis by SecA. Apyrase, which destroys nucleoside triphosphates, completely blocks Sec-dependent transport when included in the transport assays [21]. Addition of apyrase did not, however, affect import of either chimeric preprotein (Fig. 2, compare lanes T and Ap) and had no effect on the import of authentic pre-23K (data not shown) as found in previous studies [8]. We conclude that the chimeric preproteins are not imported exclusively by the Sec pathway. Transport by the ΔpH-dependent pathway is saturable [22]. Thus, precursor import can be blocked by a large excess of a second ΔpH-dependent pathway precursor. Import of the FdnG-23K precursor is completely blocked, and the TorA-23K precursor almost completely blocked, by competitor pre-23K (Fig. 2, compare T and com). This demonstrates that import of these proteins is mediated by the ΔpH-dependent pathway.

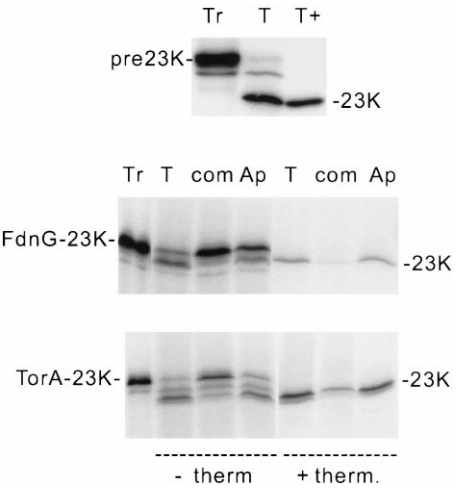

Fig. 2. Import of in vitro-translated precursor proteins into isolated pea thylakoids. The precursor proteins tested are wheat pre-23K (top panel) and the FdnG-23K (middle panel) and TorA-23K (bottom panel) chimeras. Translated, radiolabelled precursor proteins (Tr) were incubated with thylakoids as detailed in Section 2 to give lanes T. Other lanes are as the standard assay (T) but with the addition of excess unlabelled pre-23K (com), apyrase (Ap), or with the thylakoids washed and treated with the protease thermolysin following the import assay to remove unimported proteins (+ therm or T+). Bands corresponding to the precursors and to mature 23K are marked. Note that the in vitro translations products each contain a minor contaminating, import-incompetent protein intermediate in size between the authentic precursor and mature proteins (pre-23K and TorA-23K) or smaller than the precursor and mature proteins (FdnG-23K).

Thylakoid transfer peptides

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Sp 23K       AQKQDDNEANVLNSGVSRRLALTVLIGAAAVGSKVSPADA
Wh 23K        AQKNDEAASDAAVVTSRRAALSLLAGAAAIAVKVSPAAA
Sp 16K           --AQQVSAEAETSRRAMLGFVAAGLASGSFVKAVLA
Ma 16K         --ASAEGDAVAQAGRRAVIGLVATGIVGGALSQAARA
Bar PSI-N     --AAAKRVQVAPAKDRRSALLGLAAVFAATAASAGSARA
Cot PSII-T   --VQMSGERKTEGNNGRREMMFAAAAAAICSVAGVATA
Ara PSII-T   --TPSLEVKEQSSTTMRRDLMFTAAAAAVCSLAKVAMA
Chl 23K        --AVVVRASGSDVSRRAALAGFAGAAALVSSSPANA
Chl 16K         --GAVVVRASGESRRAVLGGLLASAVAAVAPKAALA
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***E. coli* transfer peptides**

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TorA                 MNNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATAAQA
FdnG                     MDVSRRQFFKICAGGMAGTTVAALGFAPKQALA
DmsA             MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFSRIAHA
PcoA                   MLLKTSRRTFLKGLTLSGVAGSLGVWSFNARS
SufI                     MSLSRRQFIQASGIALCAGAVPLKASA
NapG                 MSRSAKPQNGRRRFLRDVVRTAGGLAAVGVALGLQQQTARA
NapA                     MKLSRRSFMKANAVAAAAAAAGLSVPGVARA
NrfC                     MTWSRRQFLTGVGVLAAVSGTAGRVVA
HyaA          MNNEETFYQAMRRQGVTRRSFLKYCSLAATSLGLGAGMAPKIAWA
HybA                      MNRRNFIKAASCGALLTGALPSVSHA
HybO             MTGDNTLIHSHGINRRDFMKLCAALAATMGLSSKAAA
BisZ                     MTLTRREFIKHSGIAAGALVVTSAAPLPA
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Fig. 1. Representative thylakoid and bacterial (*E. coli*) twin-arginine transfer peptides. The hydrophobic region is underlined. Residues in bold are (i) basic residues in the C-region, (ii) the mutationally defined R-R-x-x-(hydrophobic)-(hydrophobic) twin-arginine motif for thylakoid transfer peptides, (iii) the consensus (S/T)-R-R-x-F-L-K twin-arginine motif for bacterial transfer peptides [16].

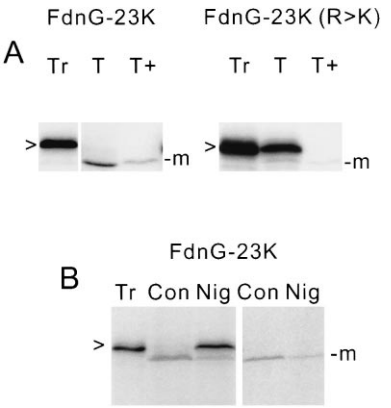

Fig. 3. A: An R5 to K5 change almost completely abolishes the ability of the FdnG transfer peptide to direct import into pea thylakoids. Import assays were performed as described for Fig. 2 using the FdnG-23K construct (left panel) and the R5K mutant on the right. Tr, translation product; T, import assay; T+, thylakoids washed and treated with thermolysin at the end of the import assay. Precursor (>) and mature (m) proteins are marked. B: Inhibition of FdnG-23K import by dissipation of the thylakoid transmembrane ΔpH gradient. Con, import assay carried out in the presence of KCl; Nig, import assay carried out in the presence of nigericin and KCl. The duplicate thylakoid assays in the right hand panel have been washed and treated with thermolysin to remove unimported proteins.

Previous studies have shown that 23K is not imported into thylakoids if fused to a chloroplast Sec pathway signal peptide [26,27]. This suggests that 23K is inherently incompatible with the Sec apparatus and reinforces the experimental conclusion that the observed import of chimeric precursors is occurring via the ΔpH-dependent pathway.

We next assessed whether the ability to mediate thylakoid import by the ΔpH-dependent pathway is a general feature of bacterial signal peptides. To do this we tested the thylakoid import capabilities of a fusion between 23K and the Sec pathway signal peptide of the *E. coli* MalE protein. The MalE-23K fusion was not imported into thylakoids (data not shown). This observation confirms that there is specificity in the interaction of bacterial twin-arginine transfer peptides with the ΔpH-dependent translocon.

Twin-arginine residues have been proposed to be an essential common feature of the targeting signals of the Sec-independent pathways of chloroplasts and bacteria [14,16]. A further test was therefore carried out to confirm that the twin-arginine motif in the bacterial transfer peptides was playing an important role in the observed thylakoid import process. The first arginine of the consensus motif in the FdnG-23K construct was changed to a lysine (R5K). This substitution, while altering the consensus motif, retains a positive charge at this position. Import assays using the R5K construct are shown in Fig. 3A. While import of the control FdnG-23K precursor is 100% efficient (Fig. 3A, left hand panel) translocation of the R5K construct is almost abolished (Fig. 3A, right hand panel). This experiment clearly demonstrates a critical role for the first arginine of the consensus motif in mediating the interaction between a bacterial transfer peptide and the plant ΔpH-dependent transport apparatus. It is, however, notable that a very low level of import (less than 2%) is still observed for the R5K construct (Fig. 3, right hand panel).

If the chimeric precursor proteins are being imported into thylakoids by the Sec-independent pathway it follows that uptake should require the thylakoid transmembrane ΔpH gradient. That this is indeed the case is demonstrated in Fig. 3B in which dissipation of the ΔpH component of the thylakoid proton electrochemical gradient by a combination of K^+ and the K^+/H^+-specific ionophore nigericin, severely inhibits uptake of FdnG-23K.

The experiments presented here demonstrate that at least two bacterial twin-arginine transfer peptides are able to mediate efficient thylakoid import by means of the ΔpH-dependent pathway, while a bacterial Sec pathway signal peptide is unable to do so. This is strong evidence that the Sec-independent protein translocases of chloroplasts and prokaryotes are mechanistically related and that proteins are directed to these systems by functionally similar targeting peptides.

The data furthermore indicate that the first arginine of the transfer peptide consensus motif is required for a bacterial transfer peptide to mediate transport by the chloroplast Sec-independent pathway. It should be noted, however, that the presence of consecutive arginine residues in the bacterial transfer peptides is unlikely in itself to be sufficient to direct protein translocation by this pathway. The Sec substrate preplastocyanin is only targeted to the ΔpH-dependent pathway with low efficiency when twin-arginine residues are placed prior to the hydrophobic region [14,26] but if the whole signal peptide is replaced by the 23K transfer peptide import efficiency is very high [12]. Thus chloroplast transfer peptides must contain targeting determinants in addition to the twin-arginine residues and these additional structural features should also be found on bacterial transfer peptides. The possible nature of these additional determinants has become clearer in the period since the first suggestion of similarity between chloroplast and bacterial twin-arginine transfer peptides [16]. A close comparison between the plant and bacterial transfer peptides (representative examples of which are shown in Fig. 1) indicates that both possess a helix-breaking residue directly before the consecutive arginine residues, and that the arginine residues are followed by a polar (predominating in bacteria) or small chain aliphatic residue, then two hydrophobic residues the second of which is usually leucine. A crucial role for the hydrophobic amino acids at either the second or third positions after the twin arginines has now been experimentally demonstrated for chloroplast transfer peptides (S. Brink, E. Bogsch, W. Edwards, P.J. Hynds and C. Robinson, submitted). A major difference between the bacterial and chloroplast transfer peptides is the identity of the amino acid at the fourth position after the twin arginines. The majority of bacterial transfer peptides have a lysine at this position. In contrast, only uncharged residues are present at the corresponding position in chloroplast transfer peptides. The effect on thylakoid import of this difference in transfer peptide structure can be assessed from the experiments above in which the import behaviour of bacterial transfer peptides with (FdnG) and without (TorA) the bacterial consensus lysine residue can be compared. No significant difference in import efficiency were observed between the constructs bearing the two transfer peptides. We can therefore conclude that the thylakoid ΔpH-dependent pathway is able to tolerate the bacterial consensus lysine residue.

It has recently been determined that the combination of twin arginine residues and a basic residue in the transfer pep-

tide C-domain prevents the 23K transfer peptide from interacting with the Sec system [26]. As the C-domain basic residue is not in itself required for translocation by the ΔpH-dependent pathway [26], and as Sec signal peptides characteristically lack basic amino acids in this region [3], this residue has been proposed to fulfil a 'Sec-avoidance' role. It is notable that the majority of bacterial twin-arginine transfer peptides also possess a basic residue in the C-region. By analogy with pre-23K these residues may be functioning as a Sec-avoidance signal in bacteria.

Clearly mutagenesis studies will be required to test the significance of the sequence similarities identified here and to identify other functionally important determinants of the Sec-independent transfer peptides.

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